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SET and MYND domain-containing protein 2 (SMYD2): a prognostic biomarker associated with immune infiltrates in cervical squamous cell carcinoma and endocervical adenocarcinoma

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Abstract: The histone lysine methyltransferase SET (Suppressor of variegation, Enhancer of Zeste, Trithorax) and MYND (Myeloid-Nervy-DEAF1) domain-containing protein (SMYD2) plays a role in the tumorigenesis of cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC). However, the prognostic significance of SMYD2 in CESC and the link between SMYD2 and tumor-infiltrating immune cells are unknown. The prognostic value of SMYD2 in CESC was obtained from The Cancer Genome Atlas (TCGA). SMYD2 mRNA and protein were both highly expressed in CESC compared with normal tissues. The high expression of SMYD2 was associated with advanced tumor status and poor prognosis in CESC patients. SMYD2 was an independent prognostic factor for overall survival. *In vitro* experiments with knockdown of SMYD2 suppressed CESC cell migration and invasion. The online tumor immune estimation resource (TIMER) and Kaplan-Meier analysis results revealed that the infiltration of CD4+ T and CD8+ T cells was related to poor prognosis. In TIMER-based multivariate Cox regression analysis, CD8+ T cells and SMYD2 were demonstrated as independent prognostic factors of CESC. In conclusion, our data suggest that high SMYD2 expression is a predictor of poor prognosis in CESC patients; SMYD2 could serve as a prognostic biomarker and molecular therapeutic target for CESC.

Keywords: cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC); SMYD2; immune infiltration

INTRODUCTION

Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) is a prevalent gynecological cancer with increased occurrence and low rate of survival [1]. According to the World Health Organization, about 570,000 women worldwide were diagnosed with CESC in 2018, and over 300,000 women died because of this disease. The most common treatments at present are surgery, radiotherapy and chemotherapy. However, many CESC patients are diagnosed at an advanced stage, which causes treatment hurdles and results in a high recurrence rate and a poor prognosis [2]. To improve the recovery rate of CESC patients, early detection and therapeutic intervention are crucial. Effective biomarkers for risk prediction, early diagnosis and prognostic evaluation of CESC are urgently needed.

The histone lysine methyltransferase SET and MYND domain-containing protein 2 (SMYD2) is capable of methylating histone H3 at lysine 4 (H3K4) or lysine 36 (H3K36) [3-5]. SMYD2 has been shown to have a crucial role in different cancer types, including gastric, colorectal and breast cancers [6-8]. A previous study also showed that SMYD2 promotes cervical cancer growth by stimulating cell proliferation [9]. However, the study had not focused deeply on exploring the underlying molecular mechanism of SMYD2 involved in the proliferation of CESC cells, and no research has been conducted to test the prognostic potential of SMYD2 gene expression level in CESC patients and the link of SMYD2 with tumor-infiltrating cells in CESC.

To investigate the comprehensive relationship between SMYD2 expression and CESC, a bioinformatics study of CESC datasets from The Cancer Genome

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Atlas (TCGA) database was performed. Differential expression of SMYD2 in CESC and normal tissues was analyzed; the correlation between SMYD2 expression and the clinicopathological characteristics and patient prognosis was investigated. Using a small interference RNA (siRNA), we induced the downregulation of SMYD2 in CaSki and SiHa cells to investigate its oncogenic effect. Finally, gene ontology (GO) analysis and gene set enrichment analysis (GSEA) were carried out to find possible biological activities and pathways of SMYD2 in CESC. The Tumor Immune Estimation Resource (TIMER) tool was used to investigate the relationship between SMYD2 expression and the tumor-infiltrating immune cells' level in CESC. Our study demonstrated that high SMYD2 expression is associated with poor prognosis of patients in CESC and that it may function as a useful prognostic indicator and a promising therapeutic target in CESC.

MATERIALS AND METHODS

Data collection

CESC clinical data and gene expression data were extracted from the online portal of TCGA (retrieved May 25, 2021), which contained 3 normal cases and 306 CESC patients. Detailed clinicopathological data, including survival status, survival time, gender, age, stage, grade, metastasis (M), lymph node (N) and primary tumor (T) were obtained in the TCGA-CESC cohort. As this study complied with TCGA publication guidelines, an ethics committee approval was not required.

SMYD2 expression analysis in CESC

The levels of SMYD2 mRNA expression in CESC and normal tissues were assessed through Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) [10]. Box plots were used to show the differential expression of SMYD2. Immunohistochemistry (IHC) staining was performed to assess SMYD2 protein expression levels in normal and CESC tissues using the Human Protein Atlas (HPA) (http://www.proteinatlas.org). The link between SMYD2 expression and clinical features such as pathological stage, T, N, M and grade was investigated using the Kruskal-Wallis and Wilcox tests.

Overall survival (OS) analysis

Cervical cancer patients in TCGA-CESC cohorts were sorted into two separate groups based on their median value of SMYD2 mRNA expression: SMYD2 low expression and SMYD2 high expression. The R "survival" package was used for comparing the OS between these groups using the Kaplan-Meier survival curve.

Analysis of ROC

The R "survivalROC" package evaluated the predictive value of SMYD2 as a CESC biomarker using receiver operator characteristic (ROC) curve analysis. The specificity and sensitivity were observed by computing the area under the curve (AUC). For evaluating the SMYD2 prognostic prediction efficiency, we computed the AUC values of 1-, 3- and 5-year survival.

Cell culture and transfection

Cervical cancer cell lines (CaSki and SiHa) were obtained from the Chinese Academy of Science Culture Collection (Shanghai, China). SiHa cells were kept at 37°C and 5% CO_2 in minimal essential medium (MEM) (Corning, NY, USA) with penicillin (100 U/mL), 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA), and streptomycin (100 U/mL). CaSki cells were cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Life Technologies, Shanghai, China) with 10% FBS, streptomycin (100 U/mL), and penicillin (100 U/mL). GenePharma (China) provided the specific siRNAs and negative control (siRNA-NC). SMYD2-siRNA, S 5'-GGGCAUUAGACCCAGAAAUTT-3'; AS 5'-AUUUCUGGGUCUAAUGCCCTT -3' were the target sequences of siRNA, and S 5'-UUC UCC GAACGUGUCACGUTT -3'; AS 5'-ACGUGACACGUUCGGAGAATT -3' were the negative control siRNA sequences. Lipofectamine 2000 (Invitrogen, MA, USA) was used to transfect SMYD2-siRNA into CaSki and SiHa cells, according to the experimental guidelines. When the cells grew to 70% confluence, qRT-PCR and Western immunoblotting (WB) was performed to assess the interference efficiency of SMYD2-SiRNA after 48 h.

RNA isolation and quantitative RT-PCR (qRT-PCR)

RNAiso Plus reagent (Takara BIO Inc, Kusatsu, Shiga, Japan) was utilized for extracting the total. The concentration and quality of RNA based on the ratio of the absorbance at 260 and 280 nm (A260/280) was determined by spectrometry. A PrimeScript RT reagent Kit (Takara) was used to prepare cDNA; qRT-PCR was performed using an ABI StepOne Real-Time PCR System and an SYBR Premix Ex Taq Kit (Takara), according to the manufacturer's instructions. The SMYD2 qRT-PCR-related forward and reverse primer sequences were 5'-TACTGCAATGTGGAGTGTCAGA-3' and 5'-ACAGTCTCCGAGGGATTCCAG -3', respectively. The expression levels of SMYD2 were measured using qRT-PCR and normalized with β-actin as the endogenous control by using the $2^{-\Delta\Delta Ct}$ method.

WB analysis

Total protein was extracted in lysis buffer after immunoprecipitation (Beyotime, China). The bicinchoninic acid protein assay kit (Beyotime) was used to determine protein concentrations. Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride filter membranes (Bio-Rad Laboratories Inc., USA). Membrane blocking was carried out using 5% non-fat milk for 3 h, followed by incubation with primary antibodies overnight at 4°C. SMYD2 (1:1000, Proteintech, IL, USA), proliferating cell nuclear antigen (PCNA, 1:2000, Cell Signaling Technology, MA, USA) and GAPDH (1:1000, Santa Cruz Biotechnology Inc., CA, USA) were the primary antibodies used. After washing, membranes were incubated at room temperature for 1 h with an anti-rabbit/mouse IgG secondary antibody (1:50000, Abcam, Cambridge, UK). Increased chemiluminescence was used to visualize protein bands, and ImageJ software was used to measure the band intensity.

Cell proliferation assay

To evaluate cell proliferation rates, the Cell Counting Kit-8 (Yeasen Biotechnology (Shanghai) Co., Ltd. China) was used. In 96-well plates of 2,000 cells per well, equal numbers of transfected CaSki and SiHa cells were plated. A microplate reader was used to assess the cell viability at 12, 24, 48, 72 and 96 h by calculating the absorbance at 450 nm. Cell proliferation was studied by conducting colony formation assays. A 6-well plate was used to plate the transfected CaSki and SiHa cells at a density of 1,000 cells per well and incubated at 37°C for 14 days under 5% CO_2 to form colonies. Colonies were then fixed using 70% methanol solution. Further, 0.1% crystal violet was used for staining and ImageJ software was used for quantification.

Migration assay

The capacity of CaSki and SiHa cell migration was measured *in vitro* 10 h after transfection using the Transwell assay. Membranes with 8-μm pores in 24-well Transwell® plates (Corning, NY, USA) were used for migration assays; 2×10^5 cells were seeded into the serum-free medium in the upper chamber 48 h after transfection, while 10% fetal bovine serum (FBS) medium was added into the lower chamber. The inserted cells were fixed in 75% methanol, stained using 0.1% crystal violet, and quantified using ImageJ software at the end of the incubation.

Multivariate and univariate Cox regression analysis

The R "survival" and R "survminer" packages were used to perform multivariate and univariate regression analyses with the aid of the Cox proportional hazard regression model to probe into the independent factors related to CESC prognosis. The hazard ratio (HR) and related 95% confidence interval (CI) were computed. Age, stage, grade and SMYD2 expression level data were included in the univariate and multivariate COX regression analyses. $P<0.05$ was considered statistically significant.

Functional enrichment analysis

The TCGA-CESC cohort was classified into two separate groups based on the median of SMYD2 mRNA expression as follows: SMYD2 high expression and SMYD2 low expression. The R "limma" package was used to identify the differentially expressed genes (DEGs) among these clusters, which included positive genes with SMYD2 (log_2FC (fold change) >0.5)

and negative genes with SMYD2 (log_2FC<-0.5), with the criteria of log2FC>0.5 and adjusted using P<0.05. The R "ggplot2" package was used to plot the volcano plots. The likely biological roles of SMYD2 positive and negative genes were investigated using GO enrichment analysis. The terms of biological process (BP), molecular function (MF) and cellular component (CC) were included in the GO analysis (MF).

GSEA is a computational method for detecting potential biological processes and signaling pathways in different gene groupings. GSEA was performed using RNA-Seq data obtained from TCGA-CESC with the reference gene set C2.cp.kegg.v7.4.symbols.gmt. For each analysis, the gene set permutations were run 1,000 times. The highly enriched pathways were identified using P<0.05 and a false discovery rate <0.25.

Immune infiltrate analysis

TIMER (https://cistrome.shinyapps.io/timer/) is an online integrative tool used for systematically analyzing the immune infiltrates in various cancer types [11]. The TIMER database was used to analyze the relationship between SMYD2 expression and immune cell infiltration, which included dendritic cells, CD8+ T cells, CD4+ T cells, B cells, macrophages, and neutrophils. P<0.05 was considered statistically significant. Kaplan-Meier curves showed the relation between tumor-infiltrating immune cells and CESC patient survival, and these were evaluated using a log-rank test. Cox analysis was employed for assessing the association between SMYD2 and these six tumor-infiltrating immune cell types.

Statistical analysis

We used R (ver. 4.0.3) for performing analysis and combining all statistical data extracted from the TCGA. The levels of SMYD2 gene expression in normal cervical tissues and CESC tissues were compared using GEPIA, and the data were assessed using one-way ANOVA. The Wilcox and Kruskal-Wallis tests were used to examine the relationship between SMYD2 expression and clinicopathological characteristics. We carried out a Kaplan-Meier analysis and the log-rank test to probe the relationship between SMYD2 expression and the OS rate of patients. Multivariate and univariate Cox regression analyses helped to assess the prognostic value of SMYD2. P<0.05 was taken as statistically significant. The link of SMYD2 to immune infiltration was observed using the purity-corrected partial Spearman correlation coefficient. All *in vitro* studies were carried out in triplicate. Data were processed using GraphPad Prism 8 software. Additionally, data were presented as the mean±standard deviation (SD) and analyzed using Student's t-test. P<0.05 was considered statistically significant.

RESULTS

SMYD2 expression analysis in CESC

The GEPIA database that was used to obtain mRNA expression data from 306 CESC and 13 normal tissues revealed that SMYD2 mRNA expression was higher in CESC tissues as opposed to unaffected tissues (Fig. 1A). The HPA database showed that SMYD2 protein levels in CESC tissues were considerably higher than in unaffected tissues (Fig. 1B and C). The link between SMYD2 expression with the clinicopathological properties of CESC patients was examined. According to our findings, the levels of SMYD2 mRNA expression were linked with CESC stages (Fig. 1D). The relationship between SMYD2 expression and T and N grades was not statistically significant (Fig. 1E, F and G). M0 had lower levels of SMYD2 expression than M1 (Fig. 1H). These outcomes showed that SMYD2 expression was related to advanced tumor status.

Patient survival and ROC analysis

Based on SMYD2 expression levels, TCGA-CESC patients were categorized into high and low cohorts. As a cut-off value, the median level of SMYD2 expression was employed. The total survival rates of the groups were compared. According to the Kaplan-Meier analysis, the SMYD2 high expression cohort had a considerably poorer OS than the low expression cohort (Fig. 1I). The diagnostic and prognostic value of SMYD2 levels in CESC was investigated further using ROC analysis. The AUCs of the SMYD2 were 0.69, 0.645 and 0.658, respectively for 1, 3 and 5 years of survival (Fig. 1J). These results highlighted that high SMYD2 expression was positively linked to poor patient prognosis, suggesting that SMYD2 could be used as a diagnostic marker in CESC.

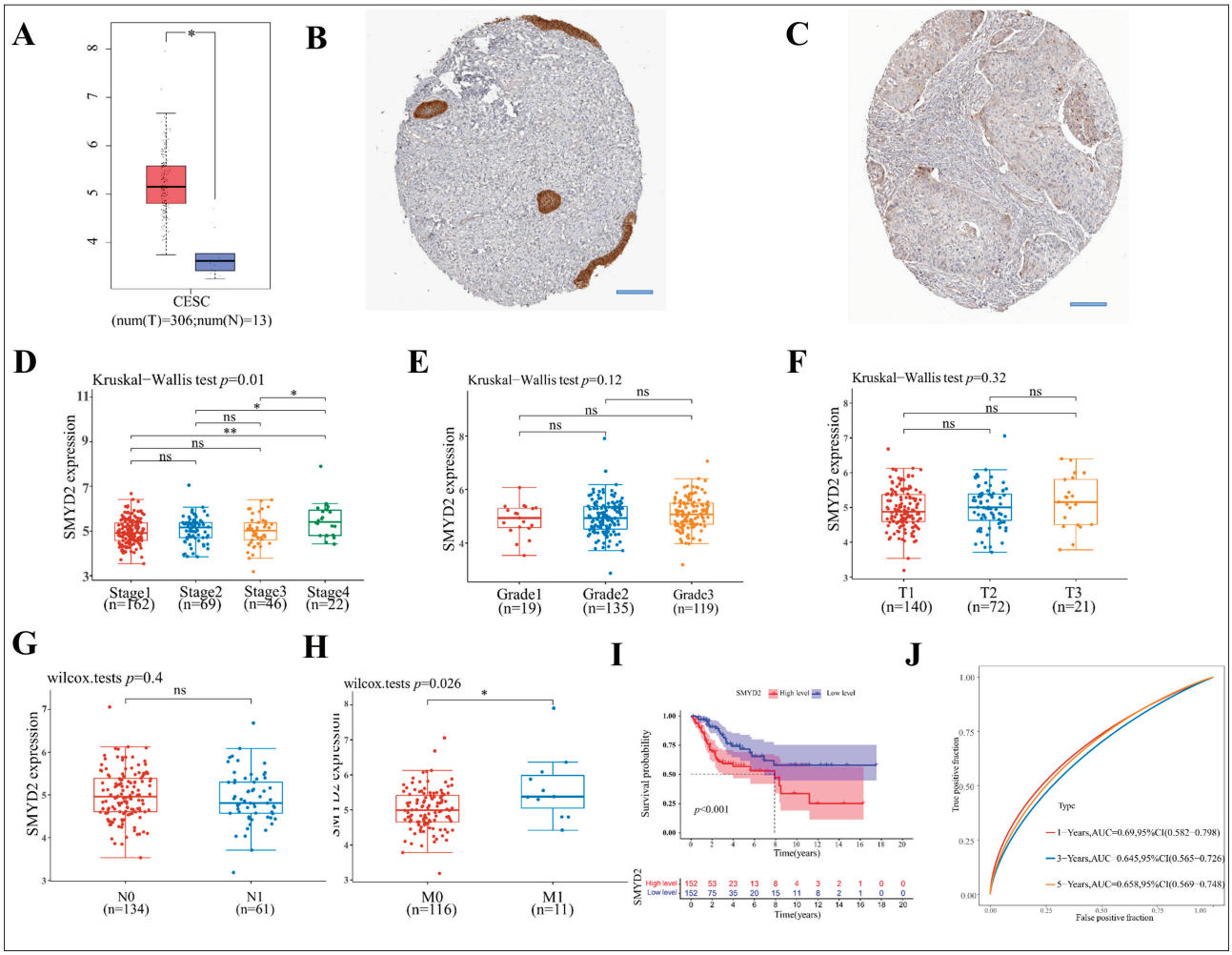

Fig. 1. Expression landscape and prognosis value of SMYD2 in CESC. **A** – Differential SMYD2 mRNA expression between CESC and normal tissues. **B**, **C** Immunohistochemical staining SMYD2 in normal (antibody HPA029023, Patient id: 1773) and CESC (Antibody HPAO029023, Patient id: 3382), respectively. **D**, **E**, **F**, **G**, **H** – Correlation between mRNA expression status of SMYD2 and clinicopathological attributes. **I** – Patients in the high SMYD2 expression cohort exhibited shorter survival times in contrast to patients in the low SMYD2 expression cohort. **J** – AUC values of ROC predicted SMYD2 corresponding to 1-, 3- and 5-year overall survival rates. CESC – cervical squamous cell carcinoma and endocervical adenocarcinoma; red: tumor tissues; blue: normal tissues. *P<0.05, **P<0.01, ***P<0.001; scale bar: 200 μm.

SMYD2 was an independent prognostic factor

Multivariate and univariate Cox regression analyses were performed to establish whether SMYD2 was an independent prognostic marker for OS in CESC. Stage (HR=1.501, 95% CI= 1.175-1.918, P=0.001) and SMYD2 (HR=1.057, 95% CI=1.025-1.090, P=0.0004) were shown to be highly linked to CESC OS based on univariate analysis (Table 1). Stage (HR=1.362, 95% CI=1.054-1.761, P=0.018) and SMYD2 (HR=1.801, 95% CI=1.166-2.783, P=0.008) were also significantly associated with OS based on multivariate Cox regression analysis. These findings showed that SMYD2 and stage were independent risk factors for CESC patient prognosis.

Table 1. Univariate and multivariate Cox regression analyses of overall survival in cervical cancer patients.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
Age	1.016	0.996-1.036	0.110			
Grade	1.037	0.678-1.586	0.868			
Stage	1.501	1.175-1.918	0.001	1.362	1.054-1.761	0.018
SMYD2	1.057	1.025-1.090	0.0004	1.801	1.166-2.783	0.008

HR – Hazard ratio, CI – Confidence interval.

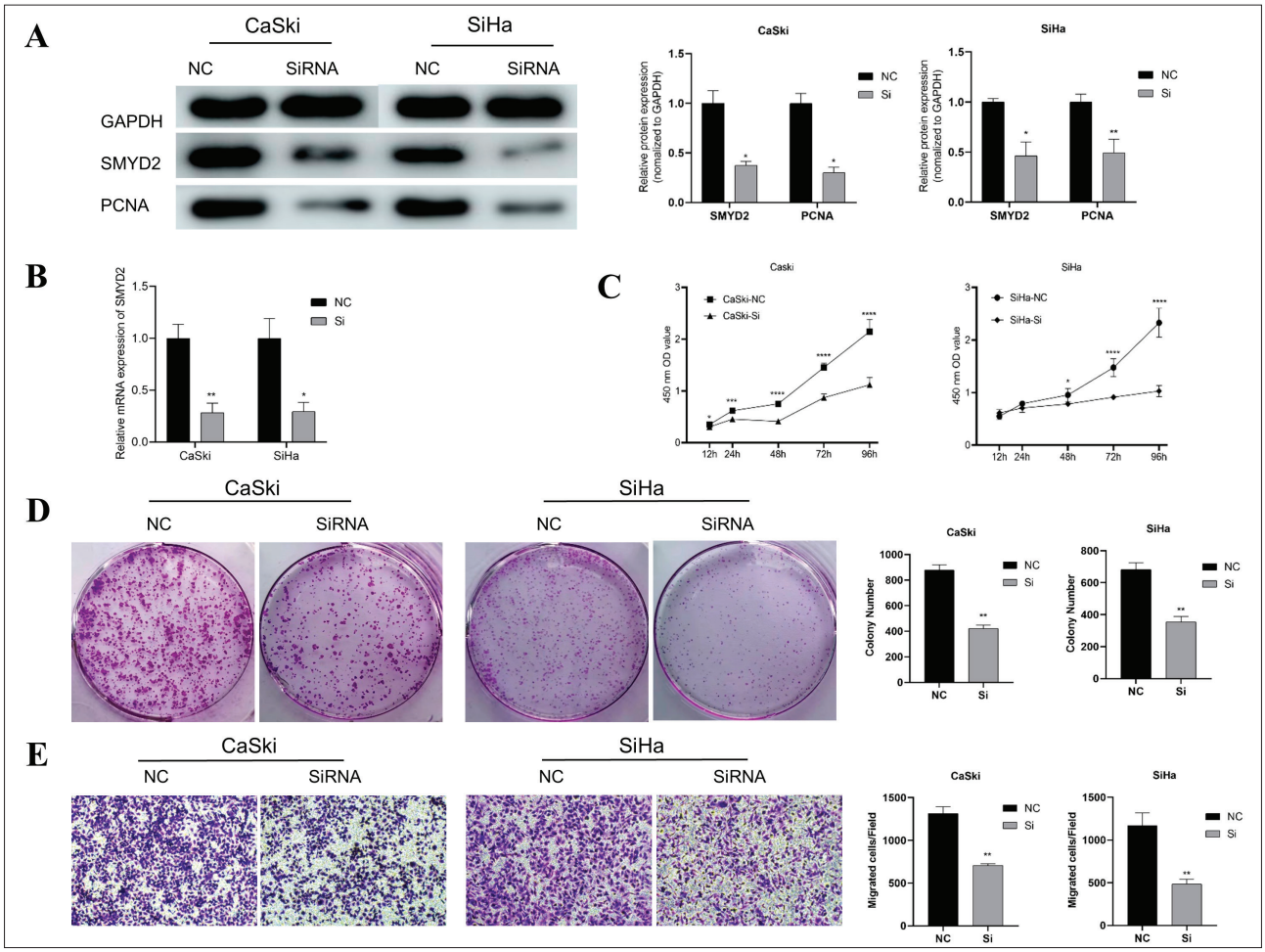

Fig. 2. Knockdown of SMYD2 expression inhibits CESC cell proliferation and migration *in vitro*. **A** – WB analysis was performed to detect the efficiency of si-SMYD2 delivery into the CaSki and SiHa cells and the expression of PCNA. **B** – qRT-PCR assay used to measure the efficiency of si-SMYD2 delivered into the CaSki and SiHa cells. **C** – SMYD2 interference inhibited cell viability in CESC cells according to the CCK8 assay. **D** – Colony formation assay used to determine the number of cell colony in CaSki and SiHa cells after transfection of SMYD2 siRNA. **E** – Transwell assay used to evaluate the migration of CaSki and SiHa cells after transfection of SMYD2 siRNA (magnification 20×). *P<0.05, **P<0.01, ***P<0.001 vs the NC group. qRT-PCR – quantitative real-time polymerase chain reaction; CCK-8 – Cell Counting Kit-8; WB – Western blot.

SMYD2-specific siRNA transfection downregulates SMYD2 expression in CESC cells

SMYD2 siRNA was developed and transfected into CaSki and SiHa cells to study the involvement of SMYD2 in CESC. Negative siRNA was used as a normal control. To demonstrate the effectiveness of siRNA delivery into cells, the expression of SMYD2 in CaSki and SiHa cells was evaluated using qRT-PCR and WB assays. When compared to siRNA-NC transfected cells, the expression level of SMYD2 in CaSki and SiHa cells was significantly reduced in both protein (P=0.0118, P=0.0151, respectively) and mRNA levels (P=0.0099, P=0.0212), and PCNA expression was reduced in si-SMYD2 of CaSki and SiHa cells (P=0.0140, P=0.0044) (Fig. 2A and B). The interference efficiency of si-SMYD2 was about 70%.

SMYD2 promotes CESC cell proliferation and migration

We evaluated the proliferation and migration status in previously established stable si-SMYD2 CaSki and SiHa cells to assess the role of SMYD2 in controlling the biological activity of CESC cells. The CCK8 and colony formation assays were used to observe how

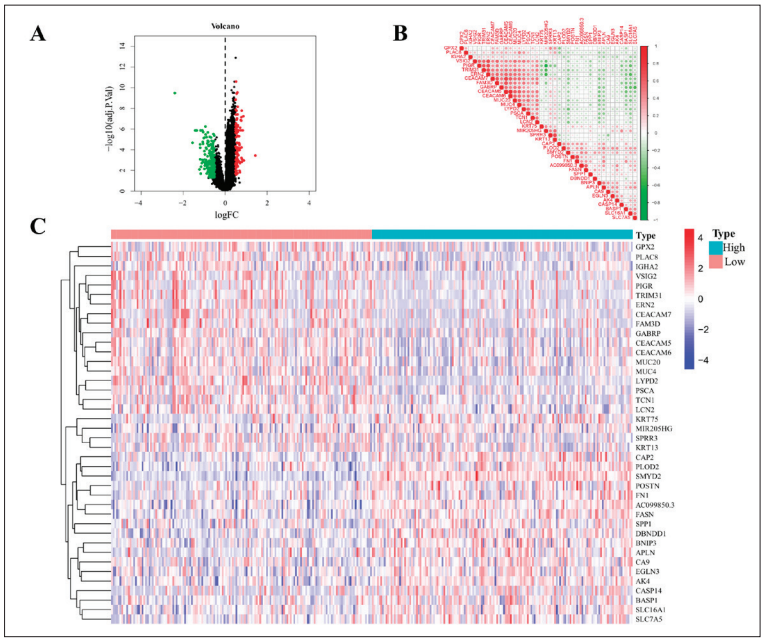

Fig. 3. DEGs between the SMYD2 high and low expression cohort. **A** – Volcano plot of the DEGs with SMYD2 high and low expression cohort. **B** – Corr-heatmap of the top 40 DEGs. **C** – Heatmap illustrating the top 40 genes negatively and positively correlated with SMYD2 in CESC. DEGs – differentially expressed genes; red color signifies positive genes; green color signifies negative genes.

SMYD2 affected cell proliferation. Compared to NC cells, CaSki-siRNA cells and SiHa-siRNA cells with low SMYD2 expression had a lower proliferation rate (P<0.0001, P<0.0001 at 96 h) and colony formation ability (P=0.0036, P=0.0049) (Fig. 2C and D, respectively). Transwell migration assays were performed to study the possible role of SMYD2 in modulating the migration ability of CESC cells. These findings revealed that downregulation of SMYD2 lowered the rate of migration in CaSki-siRNA cells and SiHa-siRNA cells compared to NC cells (P=0.0073, P=0.0083) (Fig. 2E). Our findings suggested that SMYD2 influenced CESC cell proliferation and migration capacities.

Functional enrichment analysis

A total of 313 genes (114 positive and 199 negative SMYD2 genes) were discovered to be differentially expressed in the SMYD2 high and low expression groups. Volcano plot and heatmap were plotted to illustrate the top 40 DEGs that were positively and negatively linked to SMYD2 (Fig. 3A, B and C). The roles and processes of DEGs were investigated using GO and KEGG pathway enrichment studies.

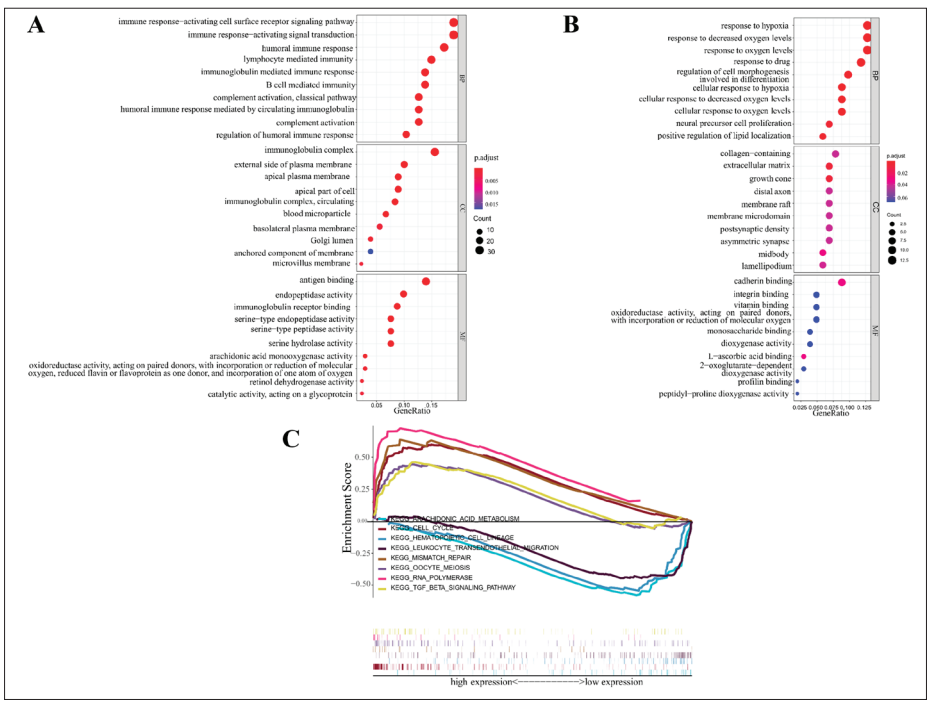

Fig. 4. Functional Enrichment Analysis. **A** – GO enrichment analysis for negative genes with SMYD2. **B** – GO enrichment analysis for positive genes with SMYD2. **C** – KEGG analysis results. GO – Gene Ontology; MF – molecular function, CC – cellular component; BP – biological process; KEGG – Kyoto Encyclopedia of Genes and Genomes.

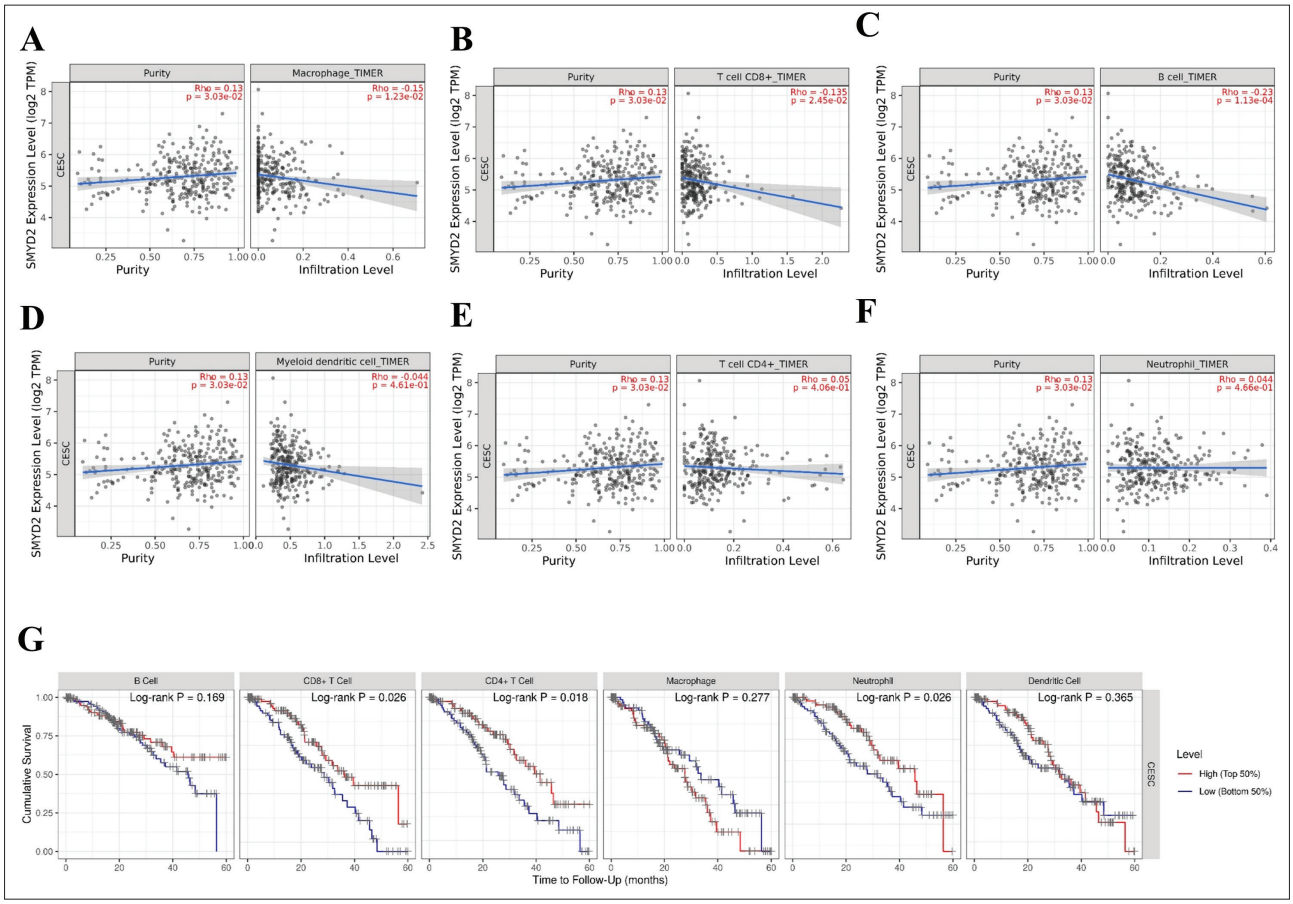

Fig. 5. Correlation analysis between SMYD2 expression and 6 kinds of infiltrating immune cells in CESC. **A**, **B**, **C**, **D**, **E**, **F** – Correlation of SMYD2 expression with 6 different types of immune infiltration cells obtained from TIMER analysis. **G** – Kaplan-Meier curve analysis illustrating the relationship between each type of immune cells and CESC prognosis.

In negative genes with SMYD2, the "immune response-activating signal transduction" and "immune response-activating cell surface receptor signaling pathway" were significantly enriched in BP. CC was mostly found in the "immunoglobulin complex" and "external side of the plasma membrane". MF had the highest levels of "antigen-binding" and "endopeptidase activity" (Fig. 4A).

In positive genes with SMYD2, the "response to hypoxia" and "response to reduced oxygen levels" were highly enriched in BP. CC was predominantly found in "collagen-containing extracellular matrix" and "growth cone". MF had higher levels of "cadherin binding" and "integrin-binding" (Fig. 4B).

KEGG analysis revealed that SMYD2 is involved in many tumor-related regulation pathways. The cell cycle, RNA polymerase, mismatch repair, oocyte meiosis and transforming growth factor (TGF-β) signaling pathway have all been linked to SMYD2 expression, which is crucial for cancer cell proliferation and progression. SMYD2 expression was found to be negatively associated with the arachidonic acid metabolism pathway, hematopoietic cell lineage and leukocyte transendothelial migration, all of which were linked to tumor suppression activities (Fig. 4C). The outcomes revealed that high SMYD2 expression is positively correlated with tumor formation in CESC.

Analysis of immune infiltration

Tumor-infiltrating immune cells are important indicators of cancer survival. The purity of a tumor is an important factor in determining cancer prognosis. TIMER was used in CESC to investigate the relationship between SMYD2 expression and

Table 2. Cox analysis of the relationship between immune cells and SMYD2 expression and prognosis in CESC patients.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
B cell	0.001	0.000-0.503	0.03	0.109	0.000-827.716	0.626
CD8+ T cell	0.037	0.002-0.755	0.032	0.006	0.000-0.827	0.042
CD4+ T cell	0.009	0.000-0.965	0.048	0.011	0.000-20.231	0.241
Macrophage	0.147	0.001-31.252	0.483			
Neutrophil	0.004	0.000-0.791	0.041	0.067	0.000-491.812	0.551
Dendritic	0.149	0.015-1.449	0.101			
SMYD2	2.046	1.426-2.934	0.000	1.94	1.346-2.797	0.000

HR – Hazard ratio, CI – Confidence interval.

tumor-infiltrating immune cell levels. SMYD2 had a strong negative correlation with the infiltration level of macrophages (r=-0.15, P=0.0123), CD8+ T cells (r=-0.135, P=0.0245) and B cells (r=-0.23, P < 0.001) (Fig. 5A, B and C, respectively). SMYD2 expression was found to have no significant association with the abundance of infiltrated dendritic cells (r=-0.044, P=0.461), CD4+ T cells (r=0.05, P=0.406) and neutrophils (r=0.044, P=0.466) (Fig. 5D, E and F, respectively). According to the Kaplan-Meier analysis, CD8+ T cells (P=0.026) and CD4+ T cells (P=0.018) can predict the outcome of CESC (Fig. 5G). These findings demonstrated a favorable association between CD8+ T cells and CD4+ T cells tumor infiltration levels and the 5-year OS rate. P<0.05 was considered statistically significant. SMYD2 (HR=2.046, 95% CI=1.426-2.934, P<0.001), B cell (HR=0.001, 95% CI=0.000-0.503, P=0.03), CD8+ T cell (HR=0.037, CI=0.002-0.755, P=0.032), CD4+ T cell (HR=0.009, CI=0.000-0.965, P=0.048) and neutrophil (HR=0.004, CI=0.000-0.791, P=0.041) were significant risks according to univariate analyses. Multivariate analyses showed that SMYD2 (HR=1.94, 95% CI=1.346-2.797, P<0.001) and CD8+ T cell (HR=0.006, 95% CI=0.000-0.827, P=0.042) were identified as independent risk factors in patients with CESC (Table 2).

DISCUSSION

CESC has become one of the most prevalent malignant gynecological tumors as evidenced by its high recurrence and mortality rate. It is still crucial to find important biomarkers linked to CESC progression that might help with early diagnosis, progression prediction and targeted treatment. Previous research has suggested that SMYD2 may have a role in a variety of cancers, acting as a tumor-enhancing gene involved in tumorigenesis and progression [12-13]. In hepatocyte carcinoma and gastric cancer, the SMYD2 expression pattern can be used as a diagnostic biomarker and a useful biomarker to inform patient prognosis [6, 14]. However, the underlying chemical process as well as the involvement of SMYD2 in CESC prognosis and the relationship between SMYD2 and tumor-infiltrating cells still needs to be studied. The objective of this study was to assess the SMYD2 expression profile for prognosis and immune infiltration in CESC patients.

In comparison with normal tissue expression levels, the SMYD2 expression level was shown to be greatly elevated in CESC at both the protein and mRNA levels. High SMYD2 expression was positively linked with advanced clinicopathological properties and poor OS in CESC. ROC curve analysis showed that SMYD2 could be used as a new diagnostic biomarker for CESC. SMYD2 was found to be an independent predictor of CESC patients based on multivariate Cox regression analysis.

We examined the oncogenic effect of CESC cells using functional assays based on bioinformatics analysis. SMYD2 had a substantial effect on CESC prognosis. The results of CCK8, transwell and WB assays revealed that SMYD2 knockdown in CaSki and SiHa cells substantially attenuated CESC cells proliferation and migration *in vitro*. Tumor cells possess malignant proliferation, migration and invasion properties that significantly influence the occurrence and progression of cancer [15]. Proliferating cell nuclear antigen (PCNA) is an important biomarker of cell proliferation [16] and its expression can indicate increased tumor cell proliferation. SMYD2 knockdown resulted in lowered PCNA expression level in CaSki and SiHa cells, where it inhibited cell proliferation according to the WB assay. The protein lysine methyltransferase SMYD2 functions as an oncogene involved in regulating its downstream target genes through histone or non-histone methylation and suppression of p53 activity to promote the metabolism of glucose [13,17]. These factors may influence tumor-cell proliferation, apoptosis, metastasis and chemosensitivity [14,18].

In this research, SMYD2 knockdown inhibited the growth of CaSki and SiHa cells, which indicated that SMYD2 acts as a tumor "instigator" in CESC.

According to the GO enrichment analysis, the negative genes associated with SMYD2 were mostly associated with anticancer immunity, including "immune response-activating signal transduction", "humoral immune response", "immune response-activating cell surface receptor signaling pathway" and "lymphocyte-mediated immune response". These immunological pathways are linked to tumors and their dysregulation is a major factor in tumor initiation and development.

Additionally, positive genes with SMYD2 were enriched in "response to hypoxia," "response to reduced oxygen levels," "response to the drug," and "regulation of cell morphogenesis involves differentiation," all of which are related to tumor development. Hypoxia is a common occurrence in cancer. The rapid proliferation of cancer cells causes the supply of nutrients and oxygen to be depleted. Hypoxia triggers a variety of adaptation pathways and genetic changes in tumor cells, allowing them to adjust to nutrition deficiency. As a result, the existence of hypoxic regions in a tumor has been linked to poor patient prognosis [19].

KEGG inquiry found that SMYD2 overexpression was linked to several tumor-related signaling pathways involving the cell cycle, mismatch repair, oocyte meiosis, RNA polymerase and TGF-β. Previous studies [20-25] have linked these pathways to cancer cell growth, metastasis and invasion activities.

Deregulated cell-cycle control is a fundamental aspect of cancer. Normal cells only proliferate in response to developmental or other mitogenic signals that indicate a requirement for tissue growth, whereas the proliferation of cancer cells proceeds essentially unchecked [21]; in this study, SMYD2 overexpression was closely linked to the cell cycle. Furthermore, experimental studies reported that the TGF-β signaling pathway is important in the advancement of cervical cancer, pelvic lymph node metastasis, proliferation and migration [26-28]. Controlling oocyte meiosis can inhibit ovarian cancer cell proliferation [29]. These outcomes support the results of the previous study showing that elevated SMYD2 expression is associated with CESC progression and poor prognosis [9]. Furthermore, increased SMYD2 expression was related to a negative relationship between the arachidonic acid metabolism pathway and hematology. The arachidonic acid pathway has a vital function in carcinogenesis [30]. Migration is one of the important characteristics of cancer cells and promotes cancer metastasis. Leukocyte transendothelial migration could make cancer cells migrate slowly at the edge of tumors, which can slow down the progression of CESC [31]. As a result, it was discovered that SMYD2 may be involved in these important pathways, but more research is required to validate this hypothesis.

SMYD2 expression was also associated with different degrees of immunological infiltration in CESC, which was an important discovery in this study. Tumor-infiltrating immune cells are involved in cancer progression and the effects of different infiltrating immune cell types vary [32]. The proportion of macrophages, CD8+ and B cells infiltrated was found to be adversely related to SMYD2 expression. According to Kaplan-Meier analysis, high CD8+ and CD4+ tumor infiltration levels had favorable associations with a 5-year OS rate, indicating that low CD8+ and CD4+ T cell infiltration was linked to poor prognosis of CESC. When compared to other variables, multivariate Cox regression analyses indicated that CD8+T cell and SMYD2 were important independent risk factors. These findings suggest that SMYD2 modulates the immunological environment and that CD8+T cell infiltration may be among the major features of CESC with prognostic value. CD8+ T cells are involved in the control of immune function such as immune surveillance of tumor cells. After stimulation by an antigen, CD8+ T cells proliferate and differentiate to cytotoxic T lymphocytes to destroy cancer cells by cell-cell interaction [33-37], indicating that the negative regulation between SMYD2 and CD8+ T cell infiltration can possibly serve as an important factor of SMYD2 with a prognostic value. However, the fundamental mechanism through which SMYD2 controls the immune response needs to be studied further.

Our findings showed that high expression of SMYD2 plays a part in the poor prognosis of CESC patients, and it can substantially increase the proliferation and migration of CESC cells. SMYD2 is a novel prognostic biomarker for CESC that may influence

immune cell infiltration. However, *in vivo* validation is required for this study. Tissues and animal experiments will be required to further investigation into the function and mechanism of SMYD2.

CONCLUSIONS

According to our findings, SMYD2 promotes cell growth and migration, exerting an oncogenic role in CESC onset and advancement. High SMYD2 expression in the CESC microenvironment is linked to poor prognosis and an increase in tumor-infiltrating immune cells. Our findings revealed that SMYD2 is a potential predictive biomarker that can also serve as a promising molecular therapeutic target for CESC. As a result, these findings provide a theoretical and experimental foundation for understanding the role of SMYD2 in cervical carcinogenesis, and that it may be a potential therapeutic target in CESC therapy.

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